Atherosclerosis

Increased Retention of LDL from Type 1 Diabetic Patients in Atherosclerosis-prone Areas of the Murine Arterial Wall

Running title: Retention of Type 1 diabetic LDL

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ABSTRACT

Background and Aims - Type 1 diabetes accelerates the development of atherosclerotic cardiovascular diseases. Retention of low-density lipoprotein (LDL) in the arterial wall is a causal step in atherogenesis, but it is unknown whether diabetes alters the propensity of LDL for retention. The present study investigated whether LDL from type 1 diabetic and healthy non-diabetic subjects differed in their ability to bind to the arterial wall in a type 1 diabetic mouse model.

Methods – Fluorescently-labeled LDL obtained from type 1 diabetic patients or healthy controls was injected into mice with type 1 diabetes. The amount of retained LDL in the atherosclerosis-prone inner curvature of the aortic arch was quantified by fluorescence microscopy. Healthy control LDL was *in vitro* glycated, analyzed for protein glycation by LC-MS/MS, and tested for retention propensity.

Results – Retention of LDL from type 1 diabetic patients was 4.35-fold higher compared to LDL from nondiabetic subjects. Nuclear magnetic resonance (NMR) spectroscopy analysis of LDL revealed no differences in the concentration of the atherogenic small dense LDL between type 1 diabetic and non-diabetic subjects. *In vitro* glycation of LDL from a non-diabetic subject increased retention compared to non-glycated LDL. LC-MS/MS revealed four new glycated spots in the protein sequence of ApoB of *in vitro* glycated LDL.

Conclusions - LDL from type 1 diabetic patients showed increased retention at atherosclerosis-prone sites in the arterial wall of diabetic mice. Glycation of LDL is one modification that may increase retention, but other, yet unknown, mechanisms are also likely to contribute.

Key Words: Type 1 diabetes; atherosclerosis; low density lipoprotein; retention

INTRODUCTION

Atherosclerosis occurs earlier in life in type 1 diabetic patients and contributes to the excess mortality of this group [**1**] but the underlying mechanisms are only partly discovered. Atherogenic dyslipidemia has been shown to be a coronary heart disease risk factor in type 1 diabetes, but the plasma level of low-density lipoprotein (LDL) cholesterol is typically not increased in type 1 diabetic patients with atherosclerotic cardiovascular disease (ASCVD) [**2**]. Factors that influence the exchange, retention and degradation of LDL in the arterial wall might therefore be particularly important determinants in the development of atherosclerosis and ASCVD in type 1 diabetic patients.

Several changes to LDL particles in type 1 diabetes could potentially lead to such differences in retention. Even modestly elevated plasma glucose concentrations in subjects with and without diabetes mellitus exacerbate ASCVD risk [**3**], and nonenzymatic glycation of apolipoprotein B (ApoB) is significantly increased in diabetic patients compared to normal subjects, even in the presence of good glycemic control [**4, 5**]. Edwards et al. demonstrated that diabetes-induced glycation of monkey LDL increased its proteoglycan-binding properties in the absence of other detectable changes in LDL composition [**6**]. Glycation of LDL may also potentiate accumulation of LDL in the arterial wall caused by decreased LDL clearance due to impaired recognition by the LDL receptor [**7, 8**]. When retained in the arterial wall, glycated LDL particles may also act more pro-inflammatory than native LDL [**9**].

Whether LDL from human diabetic patients exhibit differences in their propensity to be retained at atherosclerosis-prone sites *in vivo* has not been thoroughly addressed. Here, we show that LDL from type 1 diabetic patients is more readily retained at atherosclerosis-prone sites of mice compared with LDL from healthy controls, and we provide proof-of-concept that glycation of LDL may be a contributing mechanism.

MATERIALS AND METHODS

For a detailed Materials and Methods section, please see the Supplementary data.

Animals

All experiments were conducted on male mice 25-27 weeks of age. Type 1 diabetic mice with a hetereozygos mutation in Ins2 (C57BL/6-Ins2Akita/J) were mated with C57BL/6J mice (both from The Jackson Laboratory, Bar Harbor, ME) at Aarhus University to generate heterozygous Ins2Akita/J-/+ (Akita) and wild-type (WT) littermate controls. Mice were genotyped according to the protocol provided by the Jackson Laboratories. Before sacrifice, mice were fasted for 6 hours and blood glucose was measured using a glucometer (CONTOUR®, Bayer Consumer Care AG, Basel, Switzerland). The maximum value (33.3 mmol/L) was used when blood glucose levels exceeded the glucometer maximum measurable value.

Animal experiments were approved by the Danish Animal Experiments Inspectorate. The principles of laboratory animal care (NIH publication no. 85–23) were followed. Mice were kept under 12 h/12 h light/dark cycles, fed regular chow and given access to food and water ad libitum.

Human subjects

Ten type 1 diabetic patients (6 men and 4 women; age range 20-50 years; duration of type 1 diabetes 14.4±2.0 years; HbA1c-level 57.5±2.8 mmol/mol) were recruited at the Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Aarhus, Denmark. Type 1 diabetes was defined according to WHO criteria as insulin dependent, ketosis prone diabetes with post-prandial plasma C-peptide concentration under 200 pmoles/mL. None of the subjects were taking medications affecting the lipid profile (such as statins, fenofibrate, etc.) and all had a total plasma cholesterol < 6 mmol/L. Seven clinically healthy individuals were used as controls, with similar age and sex distributions as the patients (5 men and 2 women; age range

24–48 years), free from any medication and with total plasma cholesterol levels < 6 mmol/L. The present study was approved by the Regional Committee on Health Research Ethics (1-10-72-48-13) and was conducted according to the Declaration of Helsinki. All participants gave written informed consent.

Plasma lipoprotein particle concentration from the type 1 diabetic patients and healthy subjects was determined at LipoScience, Inc. (Raleigh, NC) by nuclear magnetic resonance spectroscopy.

Collar placement in mice

Two weeks before infusion of LDL, Akita mice were anesthetized with sevoflurane (5%) and buprenorphine (0.1 mg/kg s.c.), and the right common carotid artery was accessed through a midline incision. A partially constrictive collar made from 1.5 mm silicone tubing with an inner diameter of 0.31 mm (HelixMark, Helix Medical Inc., CA USA) was positioned around the distal part of the CCA to create local atherogenic flow, matrix proteoglycan changes, and atherosclerosis susceptibility in the immediate proximal segment as previously described [**10, 11**]. Postoperative analgesia (Rimadyl vet, 5 mg/kg s.c each day) was provided for three days.

Purification, labeling and infusion of LDL

Blood from non-fasting type 1 diabetic patients and controls was collected into vacutainer tubes containing EDTA and centrifuged at 3,000 g for 10 min at 4°C. LDL was isolated by ultracentrifugation and labeled with Atto 565 (Sigma-Aldrich, Copenhagen, Denmark) as described in detail in the Supplementary Methods and previously published [**11**]. Efficiency of Atto 565 labeling of type 1 diabetic LDL (n=6) and control LDL (n=7) was determined in a microplate fluorescence reader (PHERAstar FS, BMG Labtech). LDL (500 µg protein per mouse) was injected in Akita mice through the tail vein. Each mouse received LDL from one

individual.

Glycation of LDL

Isolated LDL from healthy individuals was diluted to 2 mg of protein per milliliter with 0.1 mol/L phosphate buffer (pH 7.4) containing 0.01% EDTA and 0.01% NaN_3 (Sigma-Aldrich). Half of the LDL preparation was incubated with 50 mmol/L D-glucose (Sigma-Aldrich) and 50 mmol/L $NaBH_3CN$ (Sigma-Aldrich) for 3 weeks at 37°C in the dark under N_2 while the other half of the LDL (control LDL) was processed identically except without the addition of glucose. The degree of glycation was estimated by the trinitrobenzenesulfonic acid (TNBSA, Pierce) assay (Thermo Scientific, Hvidovre, denmark). Glycated LDL and non-glycated control LDL was conjugated with Atto 680 NHS ester fluorochrome (Sigma Aldrich) and injected to groups of Akita and WT mice as described above.

Peptide mapping of ApoB100 modifications in *in vitro* glycated LDL

Total glycation adducts of different types was quantified in *in vitro* glycated LDL from above by peptide based proteomics using liquid chromatography-tandem mass spectrometry (LC-MS/MS). LDL was prepared according to Rabbani et al [**12**]. Subsequently, protein extracts of six samples (controls, n=3; glycated with glucose, n=3) were digested by chymotrypsin for at least 8 h (pH 7.8, 25°C) and peptides purified and analysed by nanoLC-MS/MS (Q-Exactive Plus, Thermo) [**13**]. Database searches against human proteome (20,197 Homo sapiens sequences from UniProt, August 2015) were performed in Proteome Discoverer 2.1 (Thermo), for identification of peptides and their modifications, and MaxQuant software for label-free quantification.

Tissue processing and LDL quantification

Mice were anesthesized with an intraperitoneal injection of pentobarbital (250 mg/kg) and lidocaine (20 mg/kg) 24 hours after LDL injection, euthanized by exsanguination, and tissues were processed as described in the Supplementary Methods. In mice injected with Atto 565 labeled LDL, the aortic arch was sectioned longitudinally and the right CCA cross-sectioned starting from the edge of the collar, both at 4 µm thickness. Retained LDL was quantified in the inner curvature of the aortic arch at 3 sites opposite to the branch points of the brachiocephalic artery, left CCA and left subclavian artery, respectively. In the right CCA, retained LDL was quantified 125 µm proximal to the proximal edge of the collar. The amount of retained Atto 565 labeled LDL in the aortic arch and CCA was analyzed by widefield fluorescence microscopy (Olympus Cell-R) in DAPI-stained sections using the Xcellence Imaging Software (Olympus) as described in detail in the Supplementary Methods. In mice injected with Atto 680 labeled LDL, the thoracic aortas were cleaned for periadventitial tissue, opened longitudinally and mounted on microscope slides. Atto 680 labeled LDL was then imaged and quantified by placing the aortas *en face* on a LiCor Odyssey Infrared Imaging System using the 700 nm channel as previously described [**11**]. To determine background levels, aortas from non-injected mice were used.

Statistical Analysis

All statistical analyses were performed using the Prism statistical software (GraphPad, San Diego, CA). Prior to analysis data was tested for normal distribution. Blood glucose, weight and total cholesterol level followed a normal distribution and were compared between groups using Student's t-tests. Two-sample nonparametric comparisons were performed using *Mann-Whitney test*. $P < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics of human participants and mice

Biochemical characteristics and BMI of type 1 diabetic patients and control subjects are shown in Figure 1 and Supplementary Table 1. Non-fasting blood glucose and BMI was significantly higher in type 1 diabetic patients compared with control subjects while total cholesterol levels did not differ between the two groups. The lipoprotein profile was analyzed by NMR spectroscopy and no significant differences in particle concentration or –size were detected between type 1 diabetic patients and control subjects except from a minute difference in average LDL diameter (21.11±0.34 nm in diabetic patients vs. 21.6±0.38 nm in healthy controls; Supplementary Figure 1). In particular, the analysis revealed no differences in the concentration of sdLDL between LDL from type 1 diabetic patients (274.4±47.8 nmol/L; n=10) and LDL from controls (263.8±55.3 nmol/L; n=7) (Fig. 2). The apolipoprotein content of isolated LDL from the two groups, as analyzed by LC-MS/MS was also similar (Supplementary Table 2).

Characteristics of Akita and WT mice in the fasting state are shown in Supplementary Table 3. Akita mice had a significantly lower body weight and higher fasting plasma glucose compared with WT mice.

Increased retention of LDL from type 1 diabetic patients in the arterial wall

We studied retention of Atto 565-labeled LDL from type 1 diabetic patients and healthy controls in Akita mice in both the natural lesion-prone inner curvature of the aortic arch and a site in the CCA that becomes lesion-prone after placement of an external collar to disturb blood flow [**10,11**]. No major differences in Atto 565 labeling properties were found between LDL from type 1 diabetic and control patients (data not shown). In the inner curvature of the aortic arch, most retained LDL was seen in the intimal layer and to a lesser degree in the medial layer in both groups (Fig. 3A-C). No retention was seen in the outer curvature of the aortic arch. We

found significantly more retained LDL from type 1 diabetic patients (n=10) compared to LDL from nondiabetic subjects (n=7) with a fold change of 4.35 (p <0.05) (Fig. 3E). No significant correlation was found between LDL retention and donor concentration of HbA1c within the group of diabetic patients (Supplemental Fig. 2). Further, there was no relationship between the number of sdLDL particles and the amount of retained LDL (p=0.36; R^2 = 0.06) (Fig. 3F). Thus it seems unlikely that HbA1c or sdLDL plays a significant role in the increased arterial retention of LDL from type 1 diabetic subjects.

Similarly, two weeks after induction of a lesion-prone site in the right CCA by placement of flow-disturbing collar, we found a similar pattern of retention as seen in the aortic arch. LDL was located both subendothelially and in the media (Fig. 4) with significant more retained LDL from type 1 diabetic patients compared to LDL from control subjects (fold change of 7.79; p <0.05).

Experiments with glycated LDL

Previous studies have reported increased glycation in LDL from diabetic patients [**4, 5**], but is has not been thoroughly tested *in vivo* how glycation of LDL affects the propensity for arterial retention.

To this end we glycated LDL from a non-diabetic subject *in vitro*, labeled with Atto 680 and injected into non-diabetic mice. Non-glycated *in vitro* LDL from the same donor, and processed similarly except for the addition of glucose, served as control. Incubation of LDL with glucose increased glycation with 9.8% fewer free lysine groups. This level greatly superseded that of LDL from diabetic patients, which gave little signal in the TNBSA assay. Experiments with the *in vitro* glycated LDL should therefore be interpreted cautiously.

Mass spectrometry was applied to characterize glycation of ApoB100 from LDL. *In vitro* glycated- (n=3) and non-glycated LDL (n=3) samples had close to identical protein

composition. The dominating protein was ApoB100, and constituted approximately 99% (98.9-99.4%) of the summed peptide signal of all detected peptides from apolipoproteins. The other apolipoproteins, detected at trace amounts, were ApoA1, ApoA2, ApoA4, ApoC1, ApoD, ApoE and ApoM.

Two carboxymethylation sites in ApoB100 were identified in both *in vitro* glycated and non-glycated LDL samples (Table 1), whereas hexose-modified peptides only were detected in glycated samples. Four sites on ApoB in *in vitro* glycated LDL were by MS identified to be glycated with hexose on lysine (Table 1). To estimate the degree of modification we used intensities from unmodified peptide peaks – from a peptide prone to be modified (KNIILPVYDKSLW, where K1 can be glycated) compared to a peptide not found to be modified (ELPTIIVPEQTIEIPSIKF) – and found that the signal decreased 16-50% indicating that up to 50% of that peptide could be modified under the applied conditions.

Retention of LDL is affected by in vitro glycation of LDL

We first tested retention of injected Atto 680 labeled glycated and control LDL in WT mice using infrared image scanning of the whole aorta, and found 8.87-fold more retained glycated LDL (n=8) than non-glycated LDL (n=8) (p <0.001) (Fig. 5B). The same experiment performed in Akita mice also revealed a significant increase in retained *in vitro* glycated LDL (n=5) compared to LDL prepared without glucose (n=5) with a fold change of 2.20 (p <0.05) (Fig. 5C). Again, retained LDL was seen along the inner curvature of the aortic arch at sites known to be susceptible to atherosclerosis. Aortic retention of non-glycated human LDL was higher in the Akita mice than in WT mice, consistent with our previous work [**14**].

DISCUSSION

The present study shows that LDL from type 1 diabetic patients is more susceptible to retention at atherosclerosis-prone sites in the murine arterial wall than LDL from non-diabetic subjects. Our experiments also show that heavy glycation of LDL can enhance the propensity of LDL to be retained in arteries, suggesting that glycation also at lower levels could be a potential contributing mechanism together with other, yet-unidentified LDL modifications.

LDL retention constitutes a critical first step in the development of atherosclerosis. Retained and modified LDL initiate cellular responses in the arterial wall that accelerate further LDL retention and lesion development [**15**]. Several studies have shown that intervening against the ability of LDL to be retained in the arterial wall can decrease atherosclerosis development in the absence of changes in LDL plasma concentrations [**16-22**].

It is believed that type 1 diabetes leads to more accumulation of LDL in the arterial wall [**14, 23-25**], but the mechanisms are not fully understood and could principally involve both changes in the arterial wall and circulating LDL particles. We have previously shown that type 1 diabetes in *Ins2*^{Akita} mice increases the propensity of the vascular wall to retain LDL [**14**]. Here we further show that type 1 diabetic human LDL are retained in the murine arterial wall with higher efficiency than LDL from healthy controls. Our findings indicate that this difference exists in the absence of differences in sdLDL levels or changes in the apolipoprotein composition. Nor is it related to overall glycemic control, as assessed by hemoglobin A1c measurements. Also, even though there was a slight change in average diameter, the difference appeared far too small to be relevant for the observed difference in retention. The mechanisms underlying the propensity of LDL from diabetic patients for binding in the murine arterial wall will require further research. Among the numerous possibilities are changes in the lipodome, and posttranslational modifications in the LDL apolipoproteins. Of these, glycation of ApoB has

been investigated previously as a pro-atherogenic LDL modification in diabetes, although its importance for *in vivo* retention has not been fully clarified.

Glycated LDL with a higher proportion of lysine-bound glucose is found in LDL of diabetic patients compared to the general population and these particles have been demonstrated to have distinct properties [**4, 6-8, 26**]. For example, increases in glycated LDL has been linked to promotion of atherogenesis through several mechanisms, such as increased LDL binding to proteoglycans in the arterial wall [**6**], induction of endothelial dysfunction [**27**] and accelerated foam cell formation in the arterial intima [**26**].

Few studies have examined the retention propensity of glycated compared to non-glycated LDL [**25, 28**]. Edwards et al. demonstrated that streptozotocin-induced diabetes in monkeys increased the proportion of glycated LDL and resulted in increased proteoglycan binding properties of LDL *in vitro* [**6**]. When the glycated population of LDL was removed, the LDL binding to proteoglycans was normalized. In rabbits, Wiklund et al. found three-fold greater aortic uptake of heavily *in vitro* glycated LDL (50% of lysines), compared to native LDL and less glycated LDL (5% of lysines) in a small study with no statistical comparisons [**28**]. Our findings add to these observations by showing that a less glycated preparation of injected LDL (≈10% of lysines derivatized) is significantly more retained in murine atherosclerosis-prone sites compared with native LDL.

This is proof-of-concept that glycation could be a contributing mechanism to the increased retention of LDL from diabetic patients that we observe. Its quantitative importance, however, cannot be inferred from our experiments. As in other studies we used supra-physiological glucose concentrations to glycated LDL, higher than those present in diabetes and for a much longer incubation period than the half-life of LDL in the circulation. This approach as in other applications provided glycated LDL that exert effects similar to those of *in vivo* glycated LDL

[**29**], but important differences cannot be excluded. Using the TNBSA assay, we were not able to detect a reduction of free lysines in LDL from diabetic patients compared with healthy controls. Because the TNBSA method is not reliable with low levels of glycation [**30**], this analysis does not contradict that a low level of glycation was present in LDL from diabetic donors, similar to that reported by others [**29**], but it shows that the level of modification was substantially less pronounced than for the *in vitro* glycated LDL. Comparisons between our study on *in vitro* glycated LDL and studies on LDL from diabetic patients should therefore be made with caution. The type 1 diabetic donors in the present study were under good glycemic control (HbA1c≤58 mmol/mol). Improved glucose control in type 2 diabetic patients has been shown to be beneficial with respect to a significantly lower binding of LDL to arterial proteoglycans [**31**]. It is therefore possible that LDL from less well-controlled type 1 diabetic patients will further increase the amount of LDL retained as well as the level of glycated LDL. Plasma clearance of LDL from diabetic rabbits or *in vitro* glycated human LDL has been examined in normal rabbits and generally found to be decreased [**7, 28, 32**]. At lower levels of glycation, however, the reported differences have been small or absent [**7**]. This suggests that although reduced clearance could contribute to the increased arterial retention that we find for LDL harvested from type 1 diabetic patients, it is unlikely to stand alone as the mediating mechanism.

Previous studies on human LDL have shown particle size to be an important factor in proteoglycan binding with sdLDL showing higher affinity than larger LDL particles [**33, 34**]. We enrolled type 1 diabetic patients with no significant difference in overall LDL cholesterol level or in the level of sdLDL compared to healthy controls, consistent with them being in good glycemic control. Also, we did not find any substantial differences in other aspects of LDL composition as measured by NMR spectroscopy or proteomics. It remains to be shown if

differences in lipid composition or other compositional or conformational changes exists and could be involved in the pro-retaining features of LDL from type 1 diabetic patients. Of particular interest, the propensity of LDL to aggregate has been shown to be an important and modifiable risk factor for the progression of human ASCVD [**35**]. This propensity is associated with increased LDL content of sphingolipids, including ceramides, which have previously been found to be elevated in type 2 diabetes [**36**]. Future studies may use the experimental system presented here to explore the importance of this pathway for LDL retention in type 1 diabetes, as well as its potential amelioration by dietary intervention.

In vitro glycated LDL was further investigated by LC-MS/MS which unfortunately was not possible for the LDL from type 1 diabetic patients due to limited amount of material. Previously, the LDL receptor-binding site and the primary binding site for proteoglycans were pinpointed to distinct amino acyl residues within site B (residues 3359-3369) [**37, 38**]. In this study, we did not find any lysine modified by hexose on site B. However, glycation of LDL may lead to structural changes exposing other proteoglycan-binding sites, which thus can participate in the binding of LDL to vessel wall proteoglycans in diabetes. Methylglyoxal adducts can to some degree be initiated by the presence of glucose [**39**]. Arginine-18 in ApoB of LDL has previously been shown to be prone to modification by the glycating agent methylglyoxyl [**40**]. In the present study, with glucose as glycating agent, no arginine methylglyoxylations were detected. Instead, we detected the more direct modification by the hexose glucose on lysines (4 different sites) and by the rearrangement product thereof, carboxymethyl at lysine (2 sites). Interestingly, we found four previously unrecognized sites of glycation of apoB in vitro, and further work needs to be conducted to investigate if these glycation sites also are displayed in LDL from type 1 diabetics and moreover, if they play a role in retention of LDL in the arterial wall in diabetes.

Conclusion

LDL from patients with type 1 diabetes as well as *in vitro* glycated LDL showed increased retention at atherosclerosis-prone sites in the arterial wall of mice. These findings may provide clues to some of the possible mechanisms by which type 1 diabetic patients are more susceptible to atherosclerosis. Further studies are needed to determine whether LDL glycation is the relevant modification driving increased binding of LDL in diabetic patients and to clarify its role in diabetic atherosclerosis.

CONFLICT OF INTEREST

None.

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AUTHOR CONTRIBUTIONS

MKH was responsible for conception and design, acquisition of data, analysis and interpretation of data and drafting the article. MBM, MK, JP and SG acquired data and made critical revision of the manuscript. SG furthermore contributed to conception and design. JFB contributed to conception and design and made critical revision of the manuscript. All gave final approval of the manuscript to be published.

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Lokki ML, Nieminen MS, Jula A, Perola M, Ylä-Herttula S, Rudel L, Öörni A, Baumann M, Baruch A, Laaksonen R, Ketelhuth DFJ, Aittokallio T, Jauhiainen M, Käkelä R, Borén J, Williams KJ, Kovanen PT, Öörni K. Susceptibility of low-density lipoprotein particles to aggregate depends on particle lipidome, is modifiable, and associates with future cardiovascular deaths. *Eur Heart J.* 2018;39:2562-73.

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FIGURES

FIGURE 1

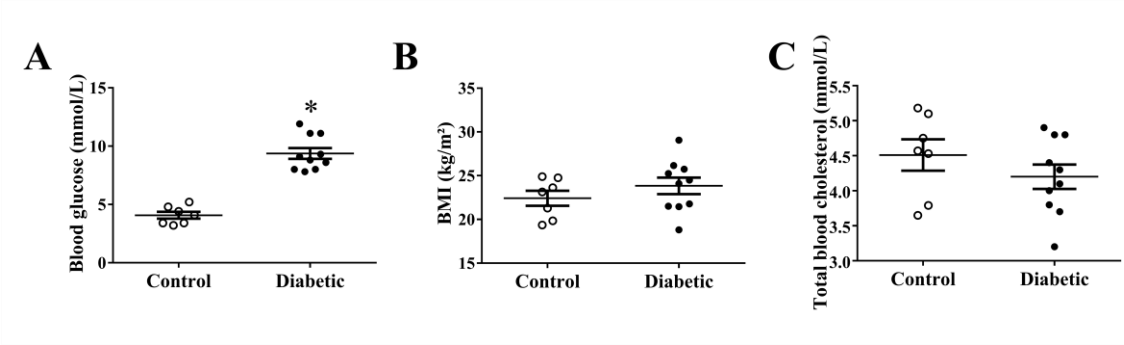


Figure 1. Characteristics of type 1 diabetic and healthy non-diabetic human subjects (controls). Type 1 diabetic patients had significantly higher blood glucose level compared to controls (A), but no significant differences were seen in BMI (B) or total blood cholesterol level (C) between groups. Controls, n=7; Type 1 diabetic patients, n=10. Asterisk indicate $p < 0.05$ (*Mann-Whitney test*). Error bars indicate SEM.

FIGURE 2

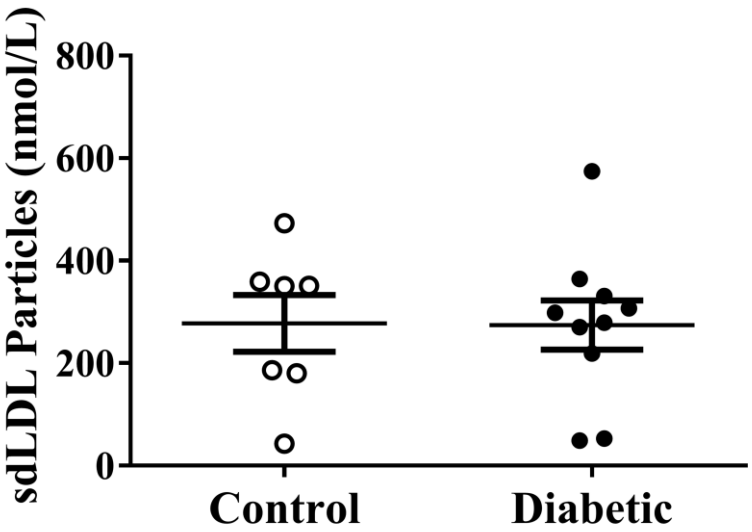


Figure 2. S**mall dense LDL (sdLDL) particle levels in type 1 diabetic patients and control subjects.** SdLDL levels were analyzed by NMR spectroscropy. No differences were seen between groups. Open symbols represent LDL from controls (n=7) and black symbols represents LDL from Type 1 diabetic patients (n=10). Error bars indicate SEM.

FIGURE 3

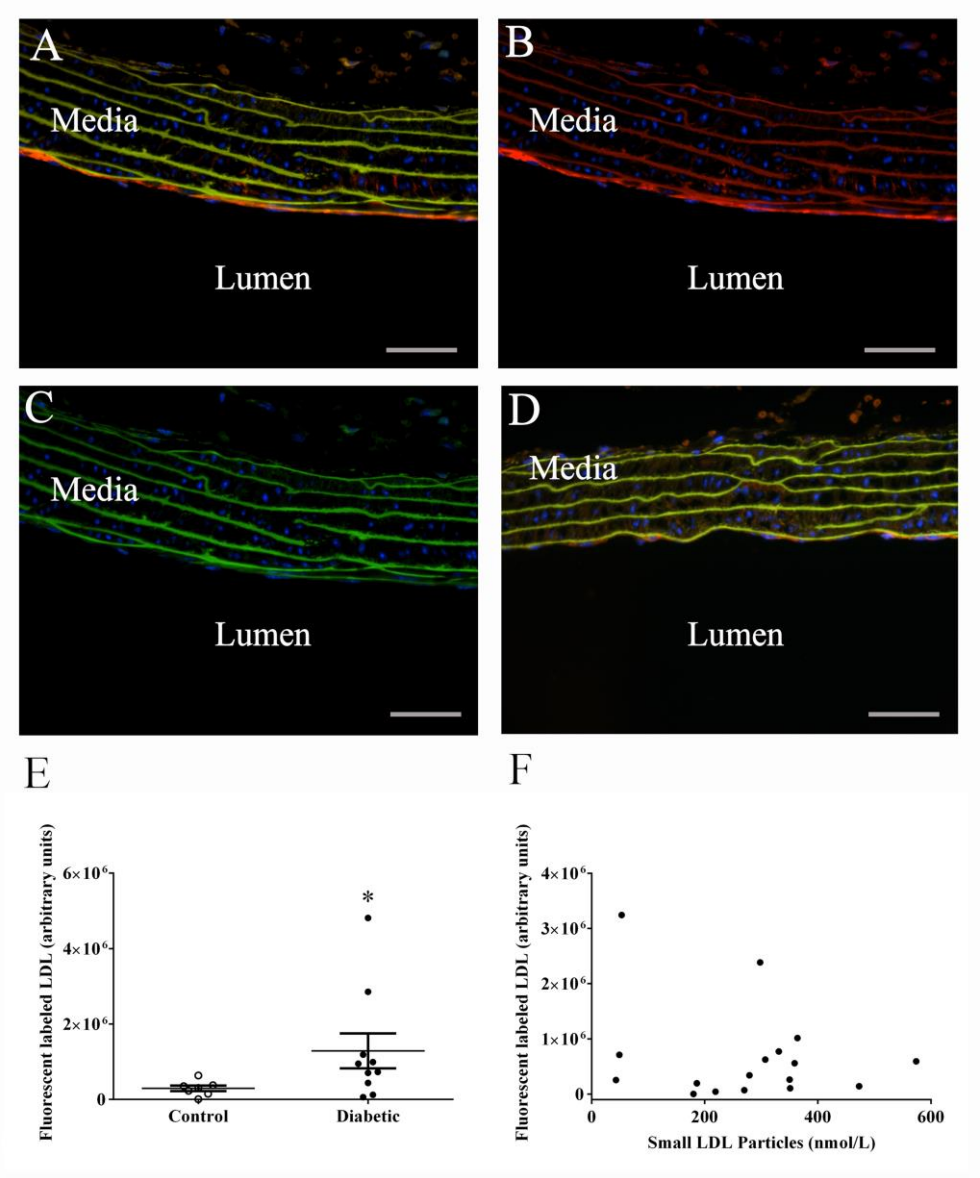


Figure 3. More low-density lipoprotein (LDL) from type 1 diabetic patients is retained in the arterial wall compared to LDL from healthy control subjects. Images show Atto 565-labeled LDL from a type 1 diabetic- (A-C) and a control subject (D) retained in the natural atherosclerosis-prone inner curvature of the aortic arch of Akita mice 24 hours after infusion. Most of the retained LDL is located in the intimal layer. The red channel (B), depicting labeled LDL and autofluorescence, and the green channel (C), depicting only autofluorescence (in particular elasitic laminae), are shown individually. (E), The amount of LDL retained in the inner curvature of the aortic arch (control, n=7; Type 1 diabetic, n=10) was calculated in ImageJ by subtracting the autofluorescence of the elastic laminae in the green channel (C) from the autofluorescence in the red channel (B). (F), No significant correlation was found between the amount of retained LDL and the level of sdLDL particles. Open symbols represents LDL from controls (n=7) and black symbols represents LDL from Type 1 diabetics (n=10). Red indicates Atto 565-labeled LDL; green; autofluorescence; blue, nuclei. Asterisk indicate $p < 0.05$ (*Mann-Whitney test*). Error bars indicate SEM. Scale-bar=50 µm.

FIGURE 4

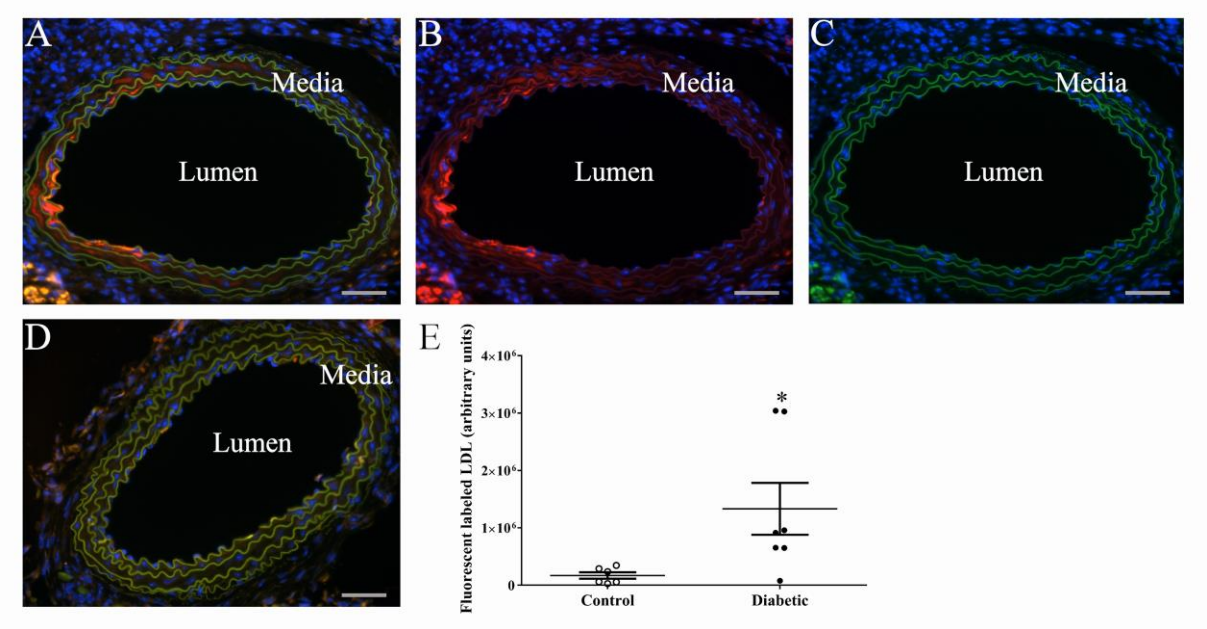


Figure 4. **Retention of LDL from type 1 diabetic patients is increased compared with LDL from healthy control subjects at induced atherosclerosis-prone sites.** Images show Atto 565-labeled LDL from a type 1 diabetic- (A-C) and a control subject (D) retained in the atherosclerosis-prone area proximal to a constrictive collar in the right common carotid artery of Akita mice, 24 hours after infusion. Most of the retained LDL is located in the intimal layer. The red channel (B), depicting labeled LDL and autofluorescence, and the green channel (C), depicting only autofluorescence (in particular elastic laminae), are shown individually. (E), The amount of LDL retained in the right common carotid artery (control, n=6; Type 1 diabetic, n=8) was calculated in ImageJ by subtracting autofluorescence of the elastic laminae in the green channel (C) from the autofluorescence in the red channel (B). Autofluorescence from tissue is seen in the lower left corner of picture (B) and (C), evident by the co-localization of the red and green channel. Red indicates Atto 565-labeled LDL; green; autofluorescence; blue, nuclei. Asterisk indicate $p < 0.05$ (*Mann-Whitney test*). Error bars indicate SEM. Scale-bar=50 µm.

FIGURE 5

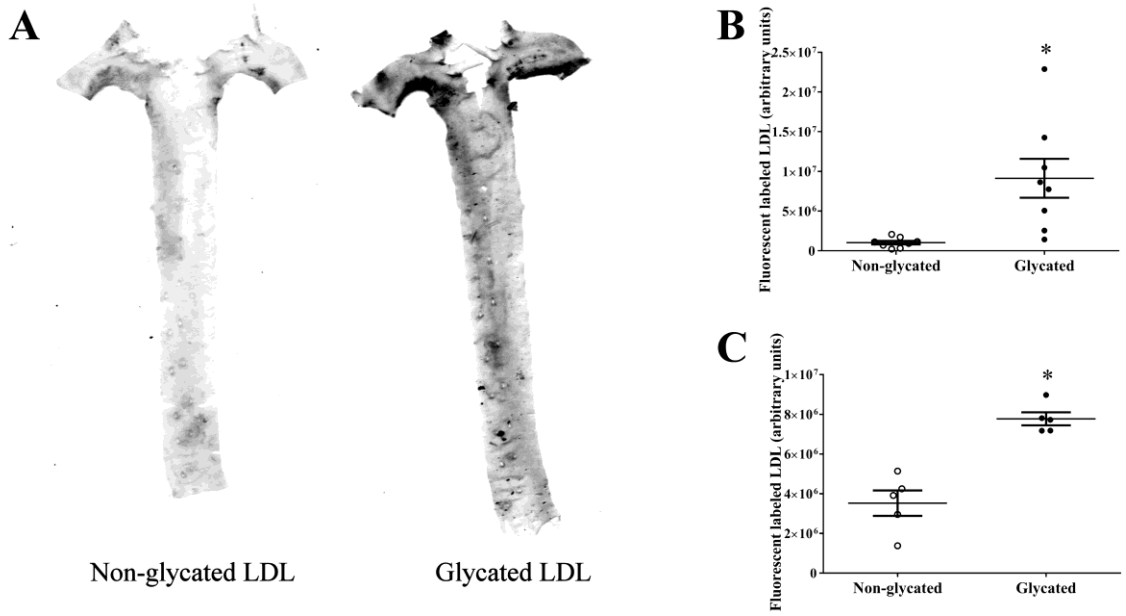


Figure 5. Glycation of low-density lipoprotein (LDL) *in vitro* increases arterial retention at atherosclerosis-prone sites of the aorta. *En face* imaging of the aorta showing retention of Atto 680-labeled LDL in the inner aortic curvature and branch points at 24 hours after infusion of glycated or non-glycated (control) LDL (A). The experiment was performed in both WT mice (n=8 in each group) and Akita mice (n=5 in each group), and the quantification of the amount of retained LDL in the two experiments are shown in (B) and (C), respectively. In the analysis of retained LDL, the intensity threshold was set so that no signal was seen in control aortas from non-injected mice. Asterisk indicate $p < 0.05$, (*Mann-Whitney test*). Error bars indicate SEM.

SUPPLEMENTARY DATA

Supplemental methods

Purification, labeling and infusion of LDL

Blood from non-fasting type 1 diabetic patients and controls was collected into Vacutainer® tubes containing EDTA and centrifuged at 3,000 g for 10 min at 4°C. LDL was isolated by ultracentrifugation at 256,000 g for 20 hours by loading plasma onto a KBr density gradient column (density layers: 1.006, 1.019, 1.063 and 1.21 g/ml). After ultracentrifugation, LDL was collected from the 1.063 g/ml density layer and desalted on a PD10 column with PBS before conjugation to Atto 565 or Atto 680 NHS ester fluorochrome (Sigma-Aldrich, Copenhagen, Denmark) at pH 8.3 for 1 hour. Excess fluorochrome was removed on a PD10 column with PBS and 24 hours before the mice were sacrificed and 500 µg/mouse labeled LDL was injected through the tail vein. Each mouse received LDL from one individual. Efficiency of Atto 565 labeling of type 1 diabetic LDL (n=6) and control LDL (n=7) was determined in a microplate fluorescence reader (PHERAstar FS, BMG Labtech).

Tissue processing and LDL quantification

Mice were anesthetized by intraperitoneal injection of pentobarbital (250 mg/kg) and lidocaine (20 mg/kg), perfused with Cardioplex and subsequently with phosphate-buffered 4% formaldehyde (pH 7.2) through the left ventricle for 1 and 5 minutes, respectively. The mice were further immersion-fixed for 6 hours at room temperature and the aortic arch and right CCA harvested. Tissues were cryoprotected in sucrose solution (25 % w/v for 24 h + 50 % w/v for 24 h), embedded in O.C.T^{TM} compound (Sakura Finetek Brøndby, Denmark) and snap-frozen in liquid nitrogen-chilled methanol:acetone (1:1). For Atto 565 quantification the aortic arch was sectioned longitudinally and

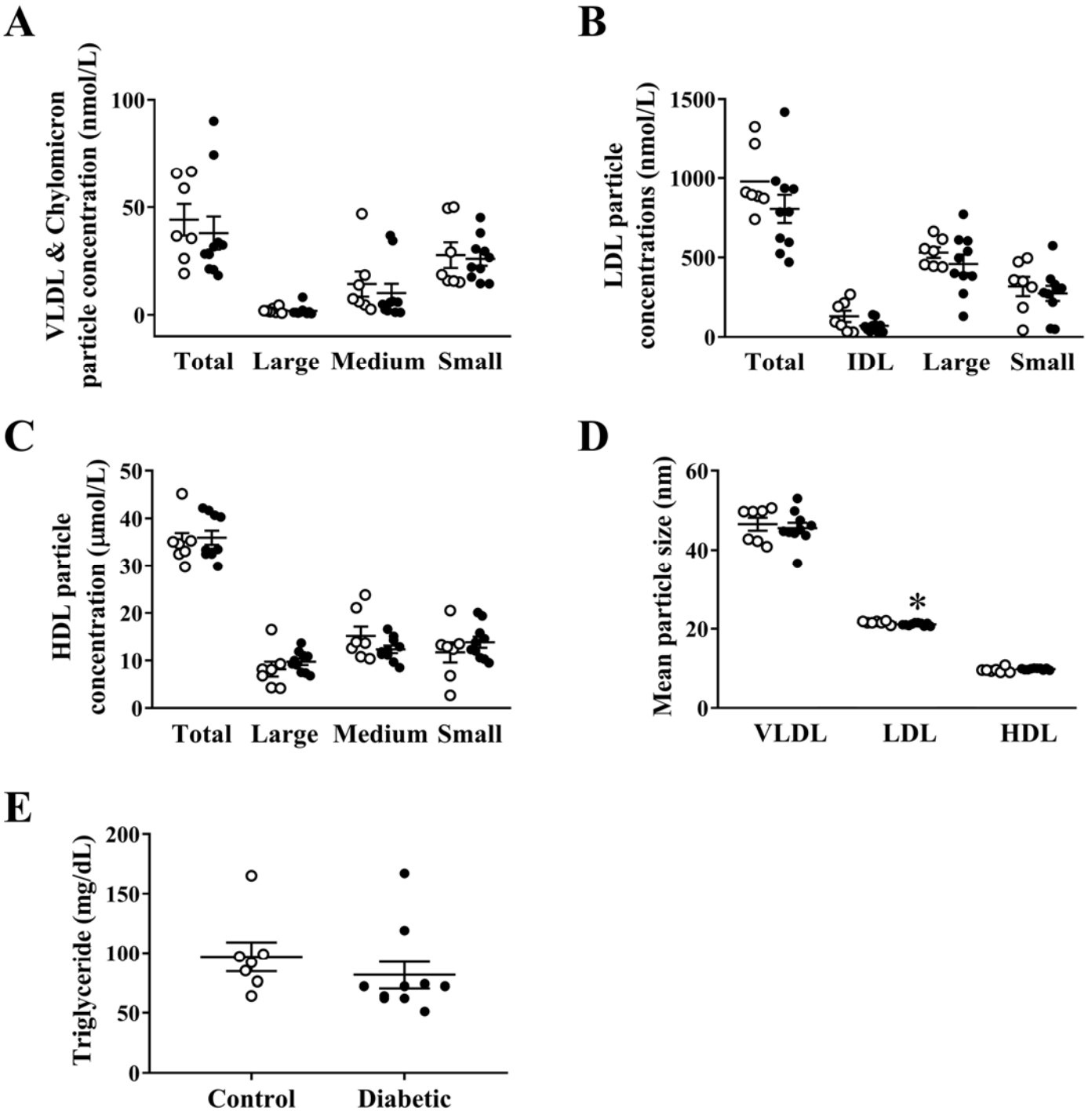
the right CCA cross-sectioned starting from the edge of the collar, both at 4 μm thickness. Retained LDL was quantified in the inner curvature of the aortic arch in 3 sites opposite to the branch points of the brachiocephalic artery, left CCA and left subclavian artery, respectively. In the right CCA, retained LDL was quantified 125 μm proximal to the proximal edge of the collar. For *en face* (frontal view) quantification of Atto 680 labeled LDL, the thoracic aortas were cleaned for periadventitial tissue, opened longitudinally and mounted on microscope slides using Aquatex (Merck) and coverslips.

To measure retained Atto 565 labeled LDL in the aortic arch and CCA using fluorescence microscopy, sections were stained with DAPI (Sigma-Aldrich), mounted with Slowfade Light Antifade (Invitrogen, Taastrup, Denmark) and analyzed for the presence of Atto 565 signal in an Olympus Cell-R widefield microscope system using the Xcellence Imaging Software (Olympus). The threshold for quantification of fluorescence signal was set by analyzing sections from 4 mice which had received an injection of saline. Retained LDL was quantified using ImageJ version 1.48v (http://rsbweb.nih.gov/ij/). Autofluorescence from the elastic laminae were circumvented by subtracting all pixel values of the green channel (autofluorescence) from the Atto 565 channel. The final intensity was calculated from the remaining signal and as the mean of intensities on the 3 images. *En face* preparations were scanned using the 700 nm channel of a LiCor Odyssey Infrared Imaging System. To determine background levels, mice which had not been injected with LDL were used.

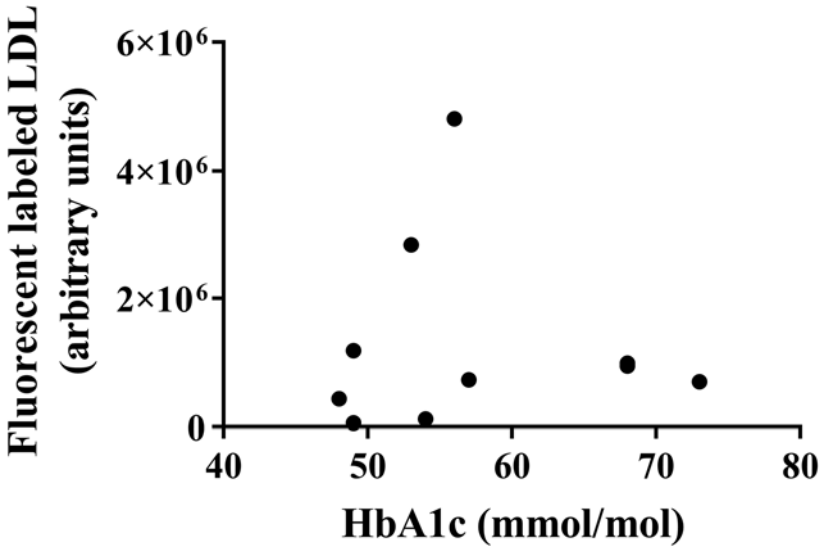
Glycation of LDL

Isolated LDL from a healthy individual was diluted to 2 mg of protein per milliliter with 0.1 mol/L phosphate buffer (pH 7.4) containing 0.01% EDTA and 0.01% NaN_3 (Sigma-Aldrich). Half of the LDL preparation was incubated with 50 mmol/L D-glucose (Sigma-Aldrich) and $NaBH_3CN$

(Sigma-Aldrich) for 3 weeks at 37°C in the dark under N_2 while the other half of the LDL (control LDL) were processed identically except without the addition of glucose. To prevent oxidation of LDL during *in vitro* glycation the incubation was done under nitrogen-flushings of the vials, to remove oxygen. All reagents were sterilized by passage through a 0.45-µm filter before incubation. At the end of incubation, free glucose was removed on a PD10 column with PBS and the concentration of LDL was measured before infusion into mice. The degree of glycation was estimated by the trinitrobenzenesulfonic acid (TNBSA, Pierce) assay (Thermo Scientific, Hvidovre, Denmark) which measures the extent of glycation indirectly, i.e., as a decrease in free nonglycated amino groups. 100 ml LDL was mixed with 100 ml of 0.1M $NaHCO_3$, pH 8.5, and 100 ml of 0.01% TNBSA. The mixture was incubated for 2 h at 37°C. 0.1 ml of 10% SDS and 0.05 ml of 1M HCL were added to stop and stabilize reaction. Absorbance of solution was measured at 335 nm. The degree of glycation was calculated from the reduction of absorbance compared with control LDL. Glycated LDL and non-glycated LDL were conjugated with Atto 680 NHS ester fluorochrome (Sigma Aldrich) at pH 8.3, purified on LD10 columns and injected into control (n=16) and type 1 diabetic mice (n=10).



Supplementary Figure 1. Lipoprotein profile of type 1 diabetic patients and non-diabetic subjects (control). Particle concentrations of (A) VLDL and Chylomicrons, (B) LDL, (C) HDL and mean particle size (D) in plasma from diabetic patients (black symbols) and non-diabetic subjects (open symbols). The triglyceride levels of diabetic and non-diabetic plasma are shown in (E). Asterisks indicate statistical significance compared to non-diabetic subjects (p <0.05; Student's t-test). Error bars indicate SEM.



Supplementary Figure 2. An increase in HbA1c in type 1 diabetic patients is not related to an increase in retention of type 1 diabetic LDL in the aortic arch of mice. Linear regression fit of data (R^2=0.0013).

Supplementary Table 1. Type 1 diabetic patients (n=10) and non-diabetic subjects (n=7) characteristics. M, men; W, women. Data presented ± SEM.

	Gender	**Age (years)**	**Blood glucose (mmol/L)**	**HbA1c (mmol/mol)**	**Total cholesterol (mmol/L)**	**BMI (kg/m^2)**
Type 1 diabetic	M=6, W=4	34.8±3.05	9.37±0.47	57.5±2.85	4.2±0.17	23.84±0.94
Non-diabetic	M=5, W=2	36.71±3.20	4.07±0.29		4.53±0.22	22.41±0.86

Supplementary Table 2. LC-MS/MS analysis of LDL from diabetic patients (n=6) and non-diabetic subjects (n=4; control). Data are presented in % ± SEM. No significant differences in apolipoproteins were found between groups (p <0.05; Student's t-test).

Gene	Control	Type 1 diabetic
APOB	97.0% ±1.0%	96.0% ±1.6%
APOM	1.0% ±0.4%	1.4% ±0.4%
APOA1	0.5% ±0.3%	1.1% ±1.0%
APOD	0.4% ±0.1%	0.4% ±0.1%
APOE	0.3% ±0.2%	0.3% ±0.2%
APOC3	0.4% ±0.2%	0.3% ±0.3%
APOA2	0.3% ±0.1%	0.2% ±0.1%
APOF	0.1% ±0.0%	0.1% ±0.1%
APOC2	0.1% ±0.0%	0.1% ±0.1%
APOC1	0.0% ±0.0%	0.0% ±0.0%
APOA4	0.0% ±0.0%	0.0% ±0.0%
APOL1	0.0% ±1.0%	0.0% ±0.0%

Supplementary Table 3. Blood glucose and weight of wildtype (WT) and Akita mice. Asterisks indicate statistical significance compared to wildtype (p <0.05; Student's t-test).

	WT mice	**Akita mice**
LDL retention / Atto 565		
Number (*n*)		17
Blood glucose (mmol/L)		28.9 ± 1.2
Weight (g)		25.2 ± 0.4
LDL retention / Atto 680		
Number (*n*)	16	10
Blood glucose (mmol/L)	7.4 ± 0.4	26.3 ± 2.2*
Weight (g)	31.5 ± 0.7	25.6 ± 0.9*